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Research article

Biodesulfurization of high-sulfur oil from the Karazhanbas field of Kazakhstan with deep eutectic solvents

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ABSTRACT

The Karazhanbas oil field in the Mangystau region of Kazakhstan contains high-sulfur oil (1.6–2.2 %). It is known that sulfur negatively affects the operational properties of petroleum products, causes the corrosion of pipelines, and adversely affects the environment and the human body. Therefore, the development of biodesulfurization technology, taking into account local features, is relevant for this field. The purpose of the study is to develop biodesulfurization of high–sulfur oil from the Karazhanbas field in Kazakhstan using deep eutectic solvents. Research objectives: isolation of sulfate-oxidizing and sulfate-reducing bacteria from the studied oils; identification of isolated bacteria; study of the effect of heavy metal Cr(VI) and sulfur on microbial activity; testing of native strains for the potential for desulfurization of crude oil. The research methodology was based on the application of the Koch methods to determine the total number of microorganisms; light microscopy – for the study of microbiological preparations; genetic identification of bacteria based on the analysis of the nucleotide sequence of a fragment of the 16S *rRNA* gene; synthesis of deep eutectic solvents; testing of isolated bacteria – for sensitivity to Cr (VI), for the ability of microorganisms to use hydrocarbons of high-sulfur oil, for activity in sulfur-containing crude oil, for determination of the mass fraction of sulfur. From 12 aerobic bacterial cultures isolated from oil samples, 9 strains with active and moderate growth in a medium with high-sulfur oil were selected during testing, followed by two strains (*Bacillus paramycoides* SFN-1, *Bacillus cereus* SFN-2), which were the most resistant to Cr (VI) and two strains (*Bacillus cereus* SFN2, *Bacillus thuringiensis* SFN3), which have shown sulfur-oxidizing abilities. The native bacterial strains selected during the study showed high disulfurization activity without the addition of deep eutectic solvents (hereinafter referred to as DES) (*Bacillus thuringiensis* SFN3), with the addition of DES-1 (*Bacillus cereus* SFN2) and with the addition of DES-2 (*Bacillus thuringiensis* SFN3). As a result of a comparative analysis of microbial desulfurization processes, it was found that the highest biodesulfurization rate at the end of the experiment was recorded in cultures of *Pseudomonas aeruginosa* B-5807 (96.3 %), *Bacillus thuringiensis* SFN-3 (96.1 %), and *Rhodococcus erythropolis* AC 1039 (96 %).

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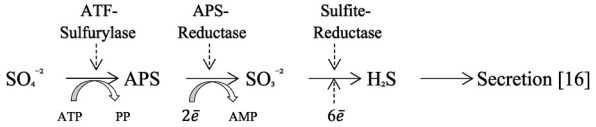
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1. Introduction

The chemical composition of crude oil varies depending on its source [1] and includes approximately 85 % carbon, 12 % hydrogen, and 3 % other compounds (oxygen, nitrogen, sulfur [2]; nickel, vanadium, etc.) [3]). Therefore, oil, as a heterogeneous hydrocarbon mixture, is divided into sulfur-, nitrogen-, and oxygen containing and organometallic [3,4].

In crude oil, the sulfur content by weight ranges from 0.03 % to 7.89 % [5], therefore, low- (up to 0.42 %) and high-sulfur (from 0.42 %) oil are distinguished [6]. At the same time, sulfur compounds in crude oil can be in two forms: inorganic (elemental sulfur S, hydrogen sulfide H_2S, carbonyl sulfide COS, dissolved pyrite FES_2) and organic (thiols RSH, sulfides R′-S-R, and thiophene compounds C_4H_4S) [7,8], which in the complex reach 50–70 % of their total content in oil [9].

Oil reservoirs belong to extreme ecosystems characterized by high temperature, salinity, and pressure [10], where the microbial component is represented by sulfur-oxidizing [11,12], nitrate-, sulfate- [] and iron-reducing [13,14] microorganisms. Of these, sulfate-reducing bacteria cause biocorrosion of oil facilities, therefore undesirable]. Under anaerobic conditions, they reduce sulfate to hydrogen sulfide in multiple stages [,15,16].



Sulfur-oxidizing bacteria aerobically oxidize sulfur compounds as electron donors to conserve energy [17] and produce byproducts of sulfate and hydrogen ions [18]:

$S^{\circ} + H_2O + 1{,}5O_2 \rightarrow SO_4^{2-} + 2H^+$, $\Delta G^{\circ\prime} = -587$ kJ/reaction [18].

As you can see, for bacteria that assimilate and utilize sulfur, the key factors are temperature, sulfur concentration [19], and water [20].

Previous studies indicate [21–25] that biodesulfurization using sulfur-oxidizing microorganisms is an environmentally friendly and cost-effective method for removing sulfur and sulfur compounds from oil and petroleum products. It should be noted that the percentage of sulfur removal from oil is influenced by local conditions and technological solutions. Therefore, our research aimed to develop a biodesulfurization method for high-sulfur oil from the Karazhanbas field in Kazakhstan using deep eutectic solvents. The research objectives included the following: isolation of sulfur-oxidizing and sulfate-reducing bacteria from the studied oils; identification of the isolated bacteria; study of the effect of heavy metals (Cr(VI)) and sulfur on microbial activity; and testing of native strains for their potential in crude oil desulfurization.

2. Materials and methods of research

2.1. Research materials

The materials for the study included.

1. Oil. Samples of crude high-sulfur oils were collected from the "Karazhanbas" fields (1.6–2.2 % sulfur [26]) and "Buzachi" (1.78 % sulfur [27]) in the Mangystau region, as well as low-sulfur oil (0.10–0.14 % by weight [28]) from the "Kumkol" field in the Ulytau region of Kazakhstan [29–31].

The oil was sterilized in a water bath at 70 °C for 15 min over 48 h. For microbiological studies, the oil samples were diluted with a solvent consisting of equal volumes of methylbenzene, acetone, and methanol, following the ISO 9120-1997 method [32].

2. Bacterial strains. Biodesulfurization experiments were conducted using identified native strains of *Bacillus cereus* (SFN2) and *Bacillus thuringiensis* (SFN3), isolated from high-sulfur oils. Additionally, strains of microorganisms *Pseudomonas putida* (B-1827), *Rhodococcus erythropolis* (AC 1039), and *Pseudomonas aeruginosa* (B-5807) were used, obtained from original cultures provided by the All-Russian Collection of Industrial Microorganisms (BRC ARCIM) [33].
3. Deep eutectic solvents (DES). Deep eutectic solvents (DES) were used to enhance the growth of microbial strains in biodesulfurization processes:
   DES-1: Composition: 2 components in a 1:2 ratio (*Bet* + *Gly* — betaine + glycerin);
   DES-2: Composition: 2 components in a 1:4 ratio (*CA* + *Gly* — citric acid + glycerin).

2.2. Research methods

2.2.1. Isolation of microorganisms

Ready-made nutrient media were used in the work: nutrient Broth and Nutrient Agar to maintain vital activity and obtain biomass of microbial cultures; Sulfur Medium for the cultivation of cyanobacteria and proteobacteria; Sulfate reducing medium for the

determination of sulfate-reducing bacteria [34]; Pseudomonas Isolation agar for the isolation and primary identification of pseudomonads [35]; mineral synthetic medium (MSS) for desulfurization [36].

The total number of microorganisms was determined by the Koch method by counting the number of colonies that grew after sowing on a solid MPA nutrient medium at a temperature of 30 °C and cultivation for 24–72 h [37]. Pure cultures were obtained by the depletion stroke method by seeding a certain volume of the studied suspension from the storage culture onto the nutrient medium with a bacteriological loop [38]. After obtaining a pure culture, experiments were conducted using electively selective media: Pseudomonas isolation agar, sulfur medium, and sulfate-reducing medium to isolate thionic and sulfate-reducing bacteria. All nutrient media were used in agarized form (2 % agar) in Petri dishes, except for sulfate-reducing medium, which was used both in liquid form (to obtain a storage culture) and in dense form (to obtain a pure culture). On dense media, inoculated material was introduced into Petri dishes in an amount of 0.1 ml; in liquid media, 8–10 % by volume, cultured at a temperature of 30 °C for 24–360 h. Aero- and anaerobic conditions were created for sulfate-reducing bacteria [39,40].

2.2.2. Microscopy methods

Microbiological preparations were prepared according to the generally accepted method of light microscopy using a binocular microscope, "Mikmed-1″ and "Motic BA 300" [41].

2.2.3. Heavy metal sensitivity test

To determine the effect of heavy metal on bacterial growth, a universal liquid medium BCH was used with the addition of sterile Cr (VI) at a concentration of 150 μg/ml (the final pH of the medium is 7) [42]. 24-hour cultures of bacteria grown on BCH were used in the experiments. Due to the different cell sizes, the optical density of daily cultures was 0.0160–0.145 units. Bacteria were cultured aerobically for 120 h at 30 °C. The crop growth was determined by the spectrophotometric method based on changes in the optical density (D_{opt}) of the medium at a wavelength of 600 nm.

2.2.4. Determination of the growth of microorganisms in an oil medium

The ability of microorganisms to use hydrocarbons of high-sulfur oil was determined on a dense medium. In a Petri dish with a sterile agarized mineral medium, a hole is cut into which the only carbon source is oil, and the cup is divided into sectors from the outside with ink on the glass. Then, each of the studied cultures of microorganisms was seeded with a loop, drawing a radial stroke about the well with a carbon source. Thick suspensions of cells are used as an inoculum, which is prepared by flushing cultures from the surface of the mowed agar. The duration of cultivation is 48 h [43].

2.2.5. Genetic identification of bacteria based on the analysis of the nucleotide sequence of a fragment of the 16S rRNA gene

DNA isolation from bacterial cultures was carried out with a set of "DNA/RNA-C-FACTOR" by the manufacturer's protocol [44]. DNA concentration and purity were assessed by measuring the optical density of micro-volumes of samples using a NanoDrop [45] 1000 spectrophotometer at 260/280 nm [46]. Fragments of the 16S rRNA genes were amplified using PCR with universal primers 8f (5′-AgAgTTTgATCCTggCTCAg-3′) and 806R (5′-ggACTACCAgggTATCTAAT-3′) in a total volume of 30 μl. The PCR mixture contained 25 ng of DNA, 1 unit of Maxima Hot Start Taq DNA Polymerase (Fermentas) 0.2 mM of each dNTP, 1 PCR buffer (Fermentas), 2.5 mM MgCl2, 10 pmol of each primer. The PCR amplification program included prolonged denaturation at 95 °C (enzyme activation and matrix denaturation) for 11 min [47] 32 cycles: 95 °C–30 s, 55°C- 40 s, 72 °C–60 s; final elongation 10 min at 72 °C [48]. DNA samples were amplified using the GeneAmp PCR System 9700 (Applied Biosystems) amplifier; the estimated operating time of the protocol is 3.5 h.

To further visualize DNA, electrophoresis was performed in a PowerPac horizontal electrophoresis chamber using a BioRad Electrophoretic Bath current source as an electrode buffer using a 1x TAE buffer. Purification of PCR products from unconnected primers and deoxynucleotide triphosphates by magnetic cleaning using magnetic silica gel [49]. The PCR product sequencing reaction was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions, followed by fragment separation on an automatic genetic analyzer 3730xl DNA Analyzer (Applied Biosystems).

2.2.6. Microbial activity in sulfur-containing crude oil

To determine the microbial activity in sulfur-containing oil, the MSS mineral medium was used, the inoculate was 24-h cultures of bacteria grown on BCH in an amount of 8 % by volume, the source of carbon and sulfur was high-sulfur oil from the Karazhanbas field, 10 % by volume. It was cultivated aerobically for 120 h at 30 °C, 160 rpm. The dynamics of the growth of microorganisms were carried out by the Koch method. The sulfur content was determined by chemical method at the beginning and at the end of the experiment [50].

2.2.7. Determination of the mass fraction of sulfur

The total mass fraction of sulfur before and after microbial desulfurization in the composition of oil was determined by the method of an energy-dispersive X-ray fluorescence sulfur analyzer, "Spectroscan S″, by regulatory documents [51], under conditions of temperature 22 °C, humidity 73 %, and pressure 695 mmHg, in the amount of a 0.1 l sample.

2.3. Biodesulfurization

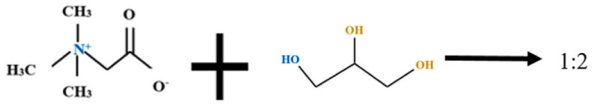
2.3.1. Preparation of the inoculum

For desulfurization, the strains of microorganisms were pre-activated on the BCH medium. To activate the lyophilized microorganisms, they were seeded on a medium of 10 ml of BCH. Bacterial strains were activated within 48–72 h [37,39].

2.3.2. Synthesis of deep eutectic solvents

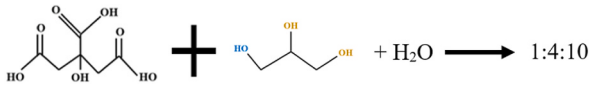
DES-1 is a mixture of betaine ($C_5H_{11}NO_2$) and glycerol ($C_3H_8O_3$) [1:2].

Synthesis of DES-1: betaine with glycerin (molar ratio 1:2) mixed under thermal conditions (50 °C) with simultaneous stirring (300 rpm) to obtain a transparent eutectic solution [52].



DES-2 is a mixture of citric acid ($C_6H_8O_7$) and glycerol ($C_3H_8O_3$) [1:4].

Synthesis of DES-2: citric acid with glycerin (molar ratio 1:4) mixed under thermal conditions (60 °C), water was added to reduce viscosity, and mixed simultaneously (300 rpm) until a transparent eutectic solution was obtained [53].



2.3.3. Experimenting

10 % inoculate and 2 % oil was added to the Mineral Synthetic medium (MSM) as the only source of sulfur. Cultivation conditions: 30 °C with constant shaking of 160 rpm on a rotary shaker for 10 days [36].

Additionally, the following control samples without microorganisms were used as controls: MSM + oil sterile medium (2 %); MSM + oil medium (2 %) + DES-1 (2 %); MSM + oil medium (2 %) + DES-2 (2 %).

To determine the effectiveness of microbial desulfurization, samples were selected for energy-dispersive X-ray fluorescence analysis.

- Determination of sulfur content (chemical analysis) – 0, 10th day;
- Determination of cell growth in fermented media - 0, 2, 5, 7, 10 days.

3. Research results and discussion

3.1. Isolation of sulfate-oxidizing and sulfate-reducing bacteria from crude oil

Table 1 shows the results of the qualitative and quantitative content of microorganisms of crude oil selected from the fields "Karazhanbas", "Buzachi", "Kumkol". As can be seen, the maximum number of microbes is observed in the crude oil of the Kumkol field, then Karazhanbas, and this indicator is an order of magnitude lower in the oil of the Buzachi field. It should be noted that all isolated microorganisms showed slow growth since colony growth was recorded only on the 3rd day of cultivation.

Thiobacteria, proteobacteria, and pseudomonas were not detected in the studied samples. The growth of both aerobic and anaerobic bacteria was recorded in the elective medium for sulfate-reducing bacteria. Sulfate-reducing bacteria (CRP) use sulfate as a terminal electron acceptor for anaerobic respiration [14,54,55]. The ability of CRP to convert sulfate into sulfide contributes to the

Table 1
Total microbial count (TMC) of crude oil, CFU/ml.

Indicator	Oil samples taken from the field		
	Karazhanbas	Buzachi	Kumkol
TMC, CFU/ml	$(1,7 \pm 0,1)*10^3$	$(2,1 \pm 0,1)*10^2$	$(6,5 \pm 0,3)*10^3$
Anaerobic sulfate-reducing bacteria	+	+	–
Aerobic sulfate-reducing bacteria	+	+	–
Thiobacteria, proteobacteria Sulfur Medium	–	–	–
Pseudomonads	–	–	–
Micromycetes	–	–	–
Actinomycetes			

Note: no growth; + there is growth.

initiation of microbiological corrosion [56]. A characteristic growth of sulfate reducers is observed on the elective medium of Sulfate-reducing medium since the blackening of media of different intensities was observed on the 7th day of anaerobic cultivation in a test tube. It is known that strict anaerobic sulfate-reducing bacteria convert sulfate into sulfide [57], therefore, when cultured on an elective medium, they react with ferrous ions to form a black color for 4–21 days at a temperature of 20–30 °C.

At the next stage of the study, pure bacterial cultures were obtained. Using the depletion stroke method on a universal MPA medium, 12 aerobic bacterial cultures were isolated from oil samples (Table 2). The purity of the isolated cultures was confirmed through visual and microscopic examination.

Morphological and cultural characteristics of the isolated cultures were studied, including colony macromorphology, cell micromorphology, Gram staining, and sporulation. Microscopic analysis revealed that 11 cultures consisted of rod-shaped bacteria (mono-, diplo-, or streptobacteria), while one culture (H6) was cocci. Of these, 11 cultures were Gram-positive, and one (H5) was Gram-negative. Additionally, nine cultures were spore-forming, whereas three (H2, H5, H6) did not form spores.

Fig. 1 shows fragments of the study of colony macromorphology; and micrographs of bacterial cells SFN1 (Fig. 1a), H2 (Fig. 1b), H5 (Fig. 1d), H1 (Fig. 1c).

The primary screening of the 12 studied microorganisms was performed on a mineral medium containing high-sulfur crude oil as the sole carbon source, using the radial cup method. Fig. 2 presents photographs illustrating the determination of bacterial strains capable of active growth in an oil-based medium. In each Petri dish containing the agarized mineral medium, a central well was filled with crude oil as the sole carbon source, and the tested bacterial strains were radially inoculated around the well (four cultures per dish).

As shown in Fig. 2, after 48 h of cultivation, bacterial growth was observed on the plates, indicating the ability to utilize carbon from high-sulfur oil. Abundant growth was noted for strains H1, H2, and H4 (Fig. 2a), while moderate growth was observed for strains F1, E1, and F2 (Fig. 2b). No growth was detected in cultures H3 and H5.

As a result of the conducted research, the following 9 crops with active and moderate growth in a medium with high-sulfur oil were selected: *SFN1, SFN2, SFN3, F1, F2, E1, H1, H2, H4*.

3.2. Genetic identification of bacteria based on the analysis of the nucleotide sequence of a fragment of the 16S rRNA gene

To identify microorganisms with active and moderate growth in a medium with high-sulfur oil, the nucleotide sequences were analyzed and combined into a common sequence in the SeqMan software (DNA Star). As a result of *16S rRNA* sequencing, all the studied samples (*SFN1, SFN2, SFN3, F1, F2, E1, H1, H2, H4*) belonged to the genera *Bacillus* and *Microbacterium* (Fig. 3).

Additionally, a phylogenetic tree was constructed with the nucleotide sequences of the *16S rRNA* gene of the reference strains of these species. The phylogenetic tree was built using the Mega X software using the Muscle algorithm to align nucleotide sequences, using the Neighbor–Joining NJ method. The reference sequences of these strains were used for the subsequent construction of a phylogenetic tree (Fig. 3).

Because of 16S rRNA sequencing and phylogenetic analysis using the Neighbor-Joining (N-J) algorithm, the isolated strains were identified with high accuracy. The isolates demonstrated 100 % identity with *Bacillus paramycoides* (SFN-1), *Bacillus thuringiensis* (SFN3), *Bacillus subtilis* (H1), *Bacillus zhangzhouensis* (F2), *Peribacillus simplex* (E−1), and *Microbacterium* sp. (H2). Additionally, 99.85 % identity was observed with *Bacillus cereus* (SFN2), *Bacillus siamensis* (H4), and *Bacillus haynesii* (F1). The obtained sequences were compared using the BLAST algorithm in GenBank, where each sequence was assigned a unique GenBank accession number.

Thus, 9 cultures of microorganisms with active and moderate growth on a medium containing high-sulfur oil as the only carbon source were identified based on microbiological and genetic methods as representatives of the genera *Bacillus, Peribacillus* and *Microbacterium*, in particular 7 cultures of bacilli: *Bacillus paramycoides* SFN-1, *Bacillus cereus* SFN-2, *Bacillus thuringiensis* SFN-3, *Bacillus subtilis* H-1, *Bacillus siamensis* H-4, *Bacillus haynesii* F-1, *Bacillus zhangzhouensis* F-2, as well as one culture of *Peribacillus simplex* E−1 and *Microbacterium* sp. H-2.

Sulfur-oxidizing, petroleum-oxidizing, hydrocarbon-oxidizing microorganisms were previously isolated from oil-contaminated soils in the oil fields of Western Kazakhstan [58]. For example, *Pseudomonas aeroginosa*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Rhodococcus erithripolis*, *Rhodococcus rubber*, *Rhodococcus maris*, *Rhodococcus luteu*, *Bacillus thuringiensis*, *Bacillus subtilis*, *Bacillus methanicus* [59]. In addition, sulfate-reducing bacteria, which are initiators of hydrogen sulphide corrosion and heterotrophic oil microorganisms, have been isolated [60]. Specifically, a high variety of sulfidogenic prokaryotes, cultures of mesophilic and thermophilic bacteria, and methanogens from the genera *Methanococcus* (5,6–25.3 %), *Methanobacterium* (up to 7 %) and *Methanothrix* (up to 2.6 %), sulfate-reducing bacteria (genera *Desulfotomaculum, Thermodesulfobacterium, Thermodesulfovibrio, Defluviitoga, Thermodesulforhabdus, Desulfonauticus* and *Desulfovirgula*) and mesophilic bacteria (genera *Desulfovibrio, Desulfomicrobium, Desulfosarcina, Desulfoglaeba, Desulfotignum* and *Desulfocurvus*), *Pseudodesulfovibrio* (31.9 %) and *Desulfocurvus* (2.4 %), as well as representatives of the genus *Enterobacter* (41.1 %) were obtained from the Karazhanbas deposit [61]. It is important to note that these cultures were taken

Table 2
Isolated cultures of microorganisms.

N⁰	Samples of oil field samples	The conditional name of the selected crops
1	Karazhanbas	H1, H2, H3, H4, H5, H6, SFN1
2	Buzachi	E1, SFN2, SFN3
3	Kumkol	F1, F2

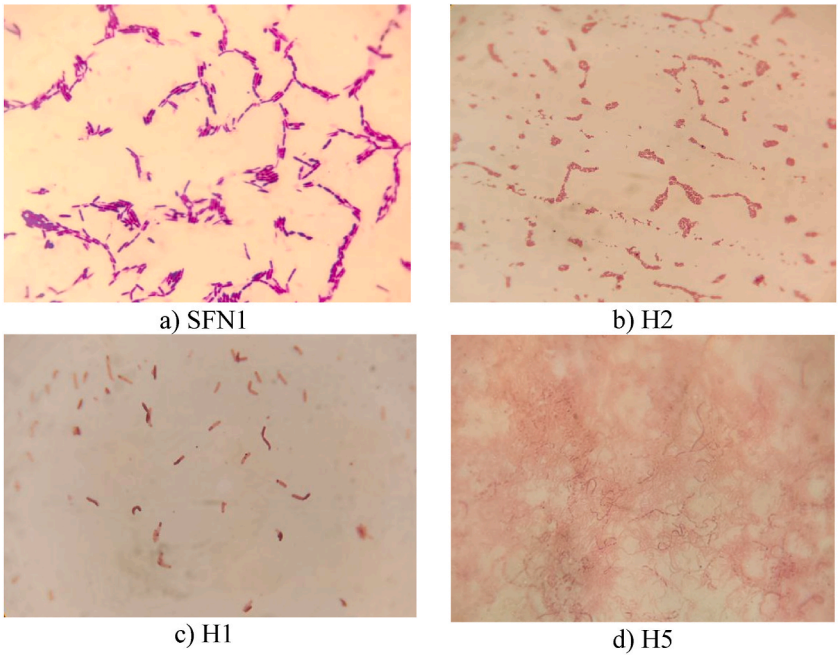


Fig. 1. Micromorphology of isolated bacterial cultures. a) SFN1; b) H2; c) H1; d) H5. The photos were taken with the SOPTOP EX330 at the magnification of 1000×.

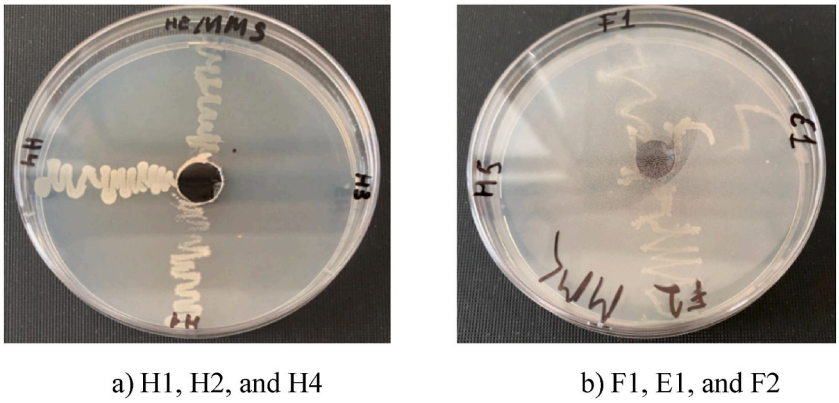


Fig. 2. Determination of bacteria capable of active growth in a medium with high-sulfur oil a) abundant growth was noted for strains H1, H2, and H4, b) moderate growth was observed for strains F1, E1, and F2.

from oily soil deposits, while in our case, they were taken from underground extreme oil systems.

The follow-up research program was aimed at investigating the effect of heavy metal on the activity of the isolated microorganisms and determining the microbial activity against sulfur in crude sulfur-containing oil.

3.3. The effect of heavy metals on the activity of isolated microorganisms

Not only pipes made of iron alloys are susceptible to corrosion, but also structures made from other alloys and even concrete. Studies have shown that tin, zinc, and lead exhibit resistance to corrosion in the presence of sulfate-reducing bacteria, possibly due to their toxic properties. Moreover, solving various environmental issues depends not only on the ability of certain microorganisms to precipitate or accumulate heavy metals but also on understanding the harmful effects of these metals on microbial communities.

The composition of oil and oil residues includes toxic heavy metals such as Cr(VI), Zn(II), and Fe(III) [62]. Consequently, the effect of heavy metals was evaluated using Cr(VI) on nine selected microbial cultures: *Bacillus paramycoides* (SFN-1), *Bacillus cereus* (SFN-2), *Bacillus thuringiensis* (SFN-3), *Bacillus subtilis* (H-1), *Bacillus siamensis* (H-4), *Bacillus haynesii* (F-1), *Bacillus zhangzhouensis* (F-2), *Peribacillus simplex* (E−1), and *Microbacterium* sp. (H-2). Control experiments were conducted by cultivating the microorganisms in BCH medium without adding Cr(VI).

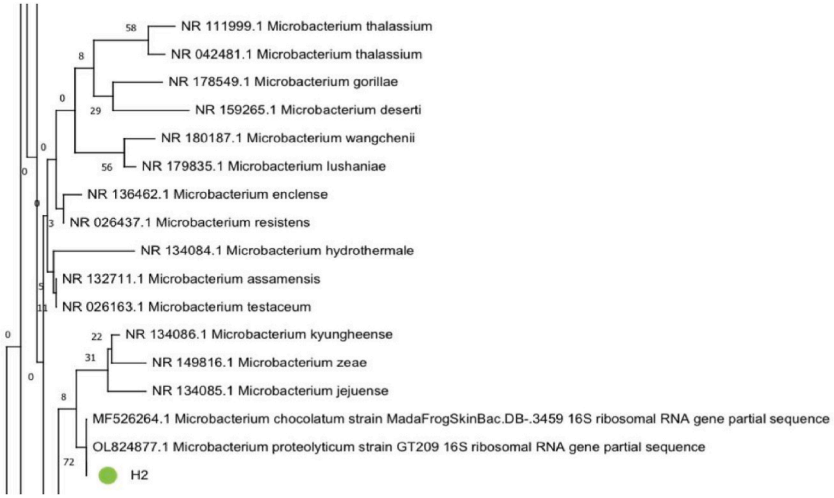


Fig. 3. Phylogenetic tree based on the analysis of the *16S rRNA* gene fragment of *SFN1, SFN2, SFN3, H1, H4, H2, F1, F2, E1* samples.

Fig. 4 shows the results of microbial cultivation on a medium with chromium at a concentration of 150 μg/ml.

A comparative analysis of the results obtained in the experiment and control indicates that the maximum increase in cells was observed.

1) 24 h of cultivation in the following cultures *Bacillus paramycoides* SFN-1 (0.287 unit in the experimental group and 0.161 unit in the control group) (Fig. 4a), *Bacillus thuringiensis* SFN-3 (0.188 and 0.776 unit respectively) (Fig. 4c), *Bacillus siamensis* H-4 (0.174 and 0.64 unit) (Fig. 4e), *Bacillus haynesii* F-1 (0.093 and 0.142 respectively) (Fig. 4f) and *Bacillus zhangzhouensis* F-2 (0.292 and 0.125 unit respectively) (Fig. 4g);
2) at different times of cultivation in the following cultures:
   – *Bacillus cereus* SFN-2: in the experiment (0.266 units) after 24 h, control (0.341 units) after 72 h of cultivation (Fig. 4b);
   – *Bacillus subtilis* H-1, *Peribacillus simplex* E−1 and *Microbacterium* sp. H-2: in the experiment (0.102 (Fig. 4d); 0.155 (Fig. 4h) and 0.15 (Fig. 4i) units, respectively) after 24 h, control (0.155 (Fig. 4d); 0.116 (Fig. 4h) and 0.157 (Fig. 4i) units, respectively) after 48 h of cultivation.

Studying the behavior of cultures in this experiment, it can be noted that the most resistant strains with relatively high cell counts in the medium with Cr(VI) were observed during 24, 48 and 72 h of cultivation, in particular, *Bacillus paramycoides SFN-1* (0.284; 0.264 and 0.213 units of cells in the medium with Cr (VI)) and *Bacillus cereus SFN-2* (0.266; 0.191 and 0.161 units of cells in a medium with Cr (VI)).

According to other studied strains, it can be seen that in the control, in a medium without Cr, there is a gradual increase in cells with a maximum cell content on the 2–3 day of cultivation, whereas, in a medium with Cr (VI), cell suppression is observed: on the first day of cultivation, the maximum number is fixed, but then the population does not develop. Literature data indicate that chromium can have both positive and negative effects on organisms. Bacteria of the genera Bacillus and *Microbacterium* can not only reduce Cr(VI), but also restore it, as noted by Campos J. (1995) [63]. From a biological point of view, chromium ions are important nutrients because they participate in electron transfer reactions in biological systems [64].

3.4. Microbial activity against sulfur in the composition of crude high-sulfur oil

To determine the potential of the studied native cultures for microbial desulfurization of selected microorganisms, the MSM mineral medium and the high-sulfur oil of the Karazhanbas field were used as a source of carbon and sulfur.

During observations of the dynamics of the growth of microorganisms on high–sulfur oil, it was revealed that the maximum increase in the number of cells was observed for culture F1 (by 3 orders of magnitude – from 10^4 to 10^7 CFU/ml), moderate cell growth (an increase of 2 orders of magnitude) - for 4 cultures (H1, F2, H4, E1), weak growth (increase by an order of magnitude) – 3 cultures (SFN1, SFN2, SFN3), H2 culture did not show growth in this medium Fig. 5 shows the results of studying bacterial growth in a medium with high-sulfur oil.

The total mass fraction of sulfur before and after microbial desulfurization in the composition of oil was determined by the method of energy dispersive X-ray fluorescence sulfur analyzer Spectroscan S, per regulatory documents [51], under conditions: temperature 22 °C, humidity 73 %, pressure 695 mmHg, in the amount of 0.1 l sample.

As a result of the chemical determination of the mass fraction of sulfur (%) in the control variants, the initial sulfur index was 1.955 %.

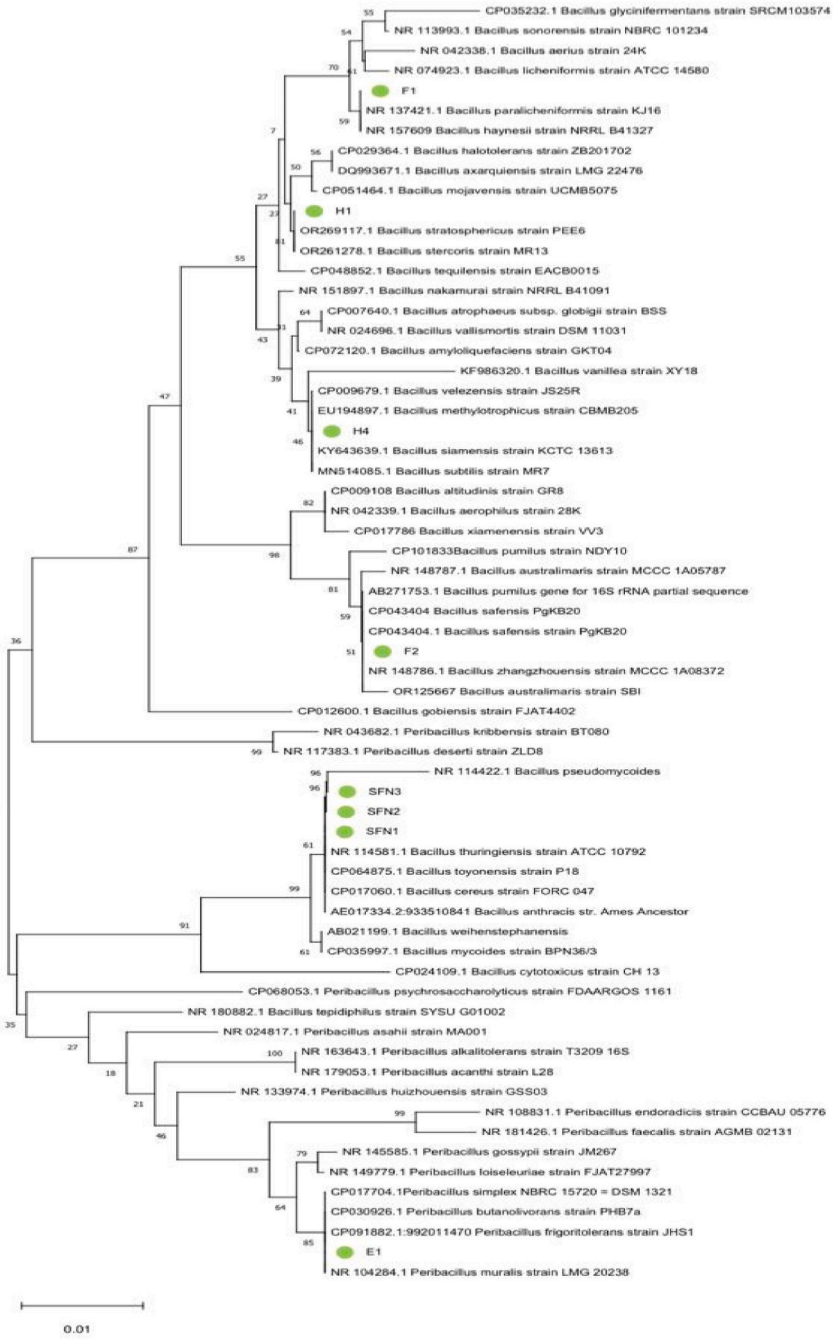
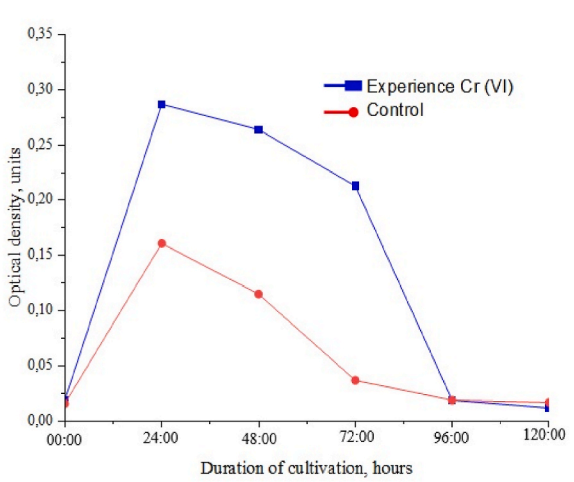


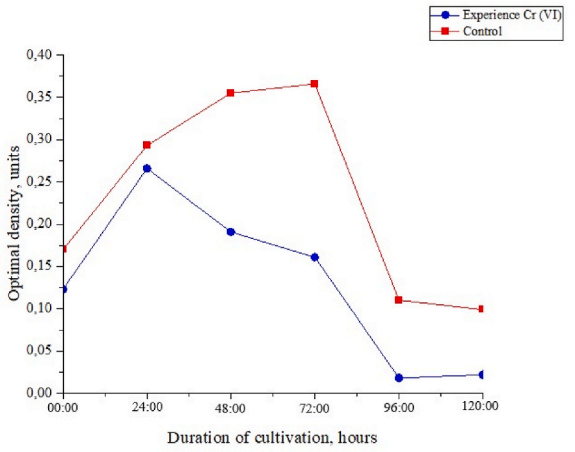
Fig. 3. (*continued*).

At the end of the experiment, on the 5th day, strains of *Bacillus cereus* (SFN2) and *Bacillus thuringiensis* (SFN3) showed sulfur-oxidizing abilities. In the experiment with *Bacillus cereus* (SFN2), the sulfur mass fraction content decreased by 0.008 %, for the experiment with the *Bacillus thuringiensis* strain (SFN3), the sulfur mass fraction content decreased by 0.364 % compared with the values obtained in the sterile oil control group (Fig. 5). For the rest of the strains we observe sulfate reducing abilities: *Bacillus paramycoides* (SFN-1) reduced sulfur to 0.182 %, *Bacillus subtilis* (H1) reduced sulfur to 0.13 %, *Bacillus siamensis* (H-4) to 0.182 %, *Bacillus haynesii* (F-1) to 0.164 %, *Bacillus zhangzhouensis* (F2) to 0.063 %, *Peribacillus simplex* (E−1) to 0.083 %, *Microbacterium* sp. (H-2) up to 0.113 % (Fig. 5).

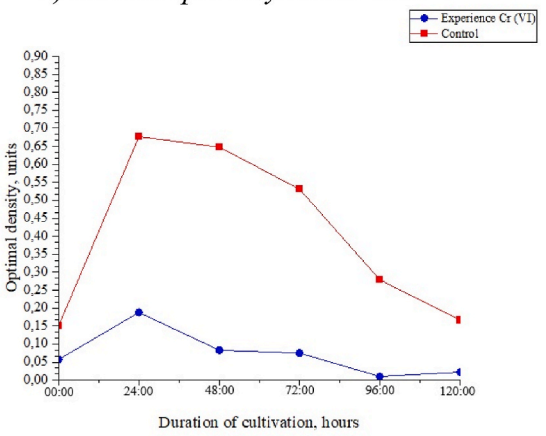
It should be noted that *Bacillus cereus* microorganisms were previously isolated and used in the works of R. A. Omar et al. for the efficient degradation of S-compounds from petroleum and petroleum products. In their studies, the technology they developed resulted



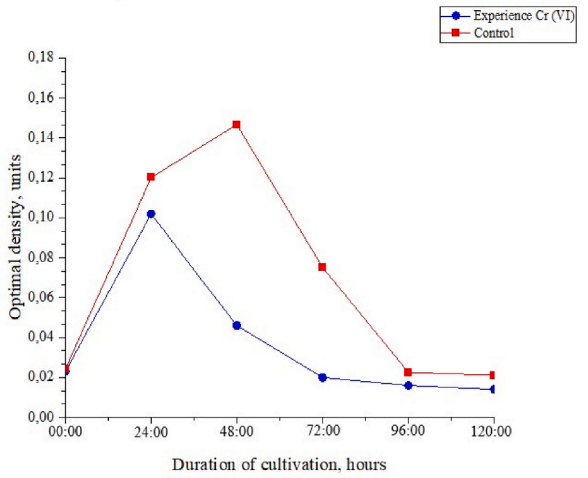
a) *Bacillus paramycoides* SFN-1



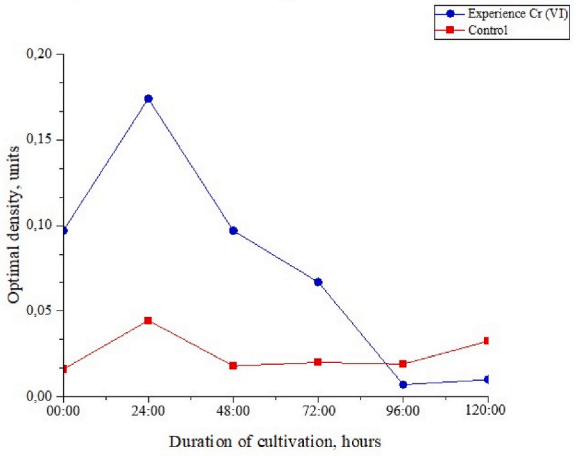
b) *Bacillus cereus* SFN-2



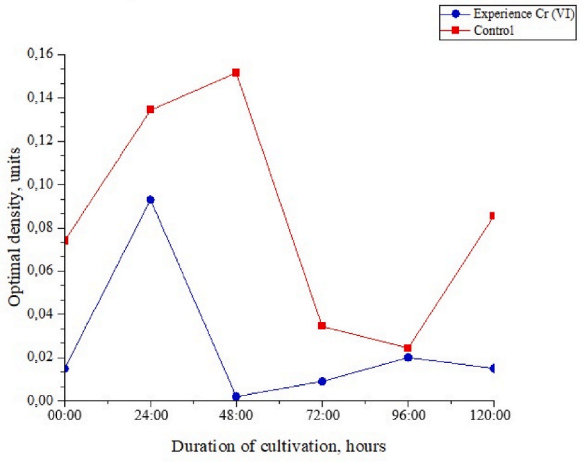
c) *Bacillus thuringiensis* SFN-3



d) *Bacillus subtilis* H-1

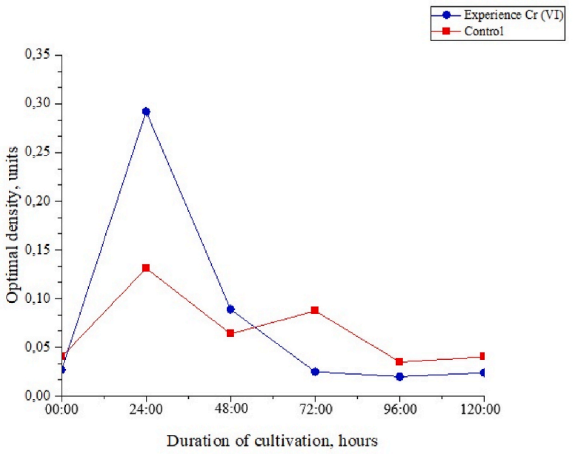


e) *Bacillus siamensis H-4*

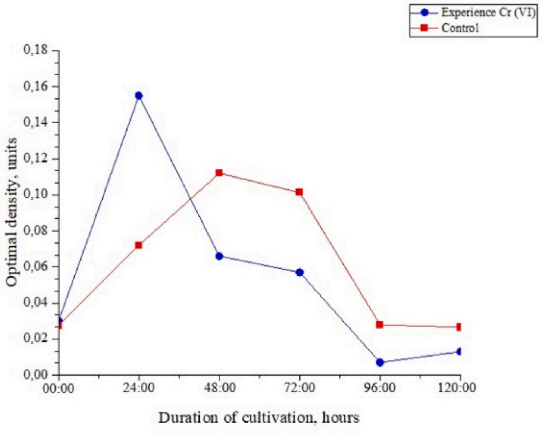


f) *Bacillus haynesii* F-1

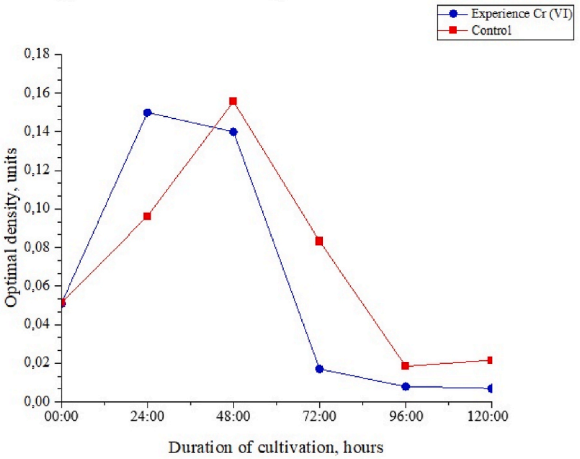
Fig. 4. Dynamics of changes in optical density (units) during the cultivation of bacteria on BCH with the addition of Cr (VI), D_{opt} (experiment) and without Cr (VI), D_{opt} (control). a) Bacillus paramycoides SFN-1 b) Bacillus cereus SFN-2, c) Bacillus thuringiensis SFN-3, d) Bacillus subtilis H-1, e) Bacillus siamensis H-4, f) Bacillus haynesii F-1, g) Bacillus zhangzhouensis F-2, h) Peribacillus simplex E-1, i) Microbacterium sp. H-2.



g) *Bacillus zhangzhouensis F-2*



h) *Peribacillus simplex E-1*



i) *Microbacterium sp. H-2*

Fig. 4. (*continued*).

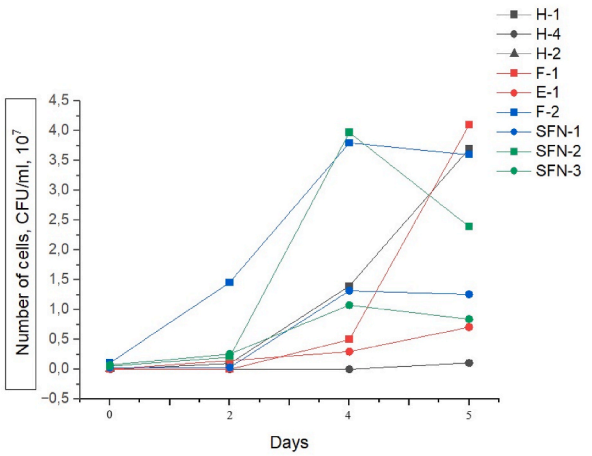


Fig. 5. Growth of microorganisms in a medium with high-sulfur oil, CFU/ml.

in 99 % desulfurization of oils containing DBT and thiophene [65], while F. Boshagh in studies with *Bacillus cereus* strain achieved 71 % in oil desulfurization [66]. In studies by M. S. Tabatabaee and M. M. Assadi, SARA ASTM D4124-01 test revealed a 65.5 % reduction in asphaltenes, 22.1 % reduction in aliphatic compounds, and a 30.3 % reduction in aromatic compounds in MSM medium. Further results with 0.9 % saline solution showed a decrease in the content of asphaltenes by 55 % and aromatic compounds by 2.1 %, respectively [67]. D. Arabian and co-authors found after 72 h of desulfurization that the total sulfur content in kerosene decreased to 1557 parts per million [68]. *B. thuringiensis* decomposed 80 % of the weight of crude oil in the works of Marwah Thamer [69]. In the studies of B. Wu and co-authors, the degradation of crude oil was carried out by joint cultivation of *Bacillus subtilis* and *Pseudomonas aeruginosa* and about 75 % of the saturated fractions of crude oil were effectively removed. These microorganisms can be metabolized and multiplied in a medium with crude oil as the only source of carbon [70]. The biosurfactant of the strain increased the decomposition of waste engine oil components of polycyclic aromatic hydrocarbons (PAHs) to 82 % in 18 days of incubation, which is more than twice as compared with decomposition without the addition of biosurfactants, recovered 85 % of used engine oil from contaminated sand in 24 h [71]. *Bacillus zhangzhouensis* bacteria also effectively removed thiol compounds from liquids. For the first time, approximately 99 % desulfurization of oil containing dibenzothiophene and thiophene was achieved and the remaining S-compounds were effectively decomposed to ~ 3–15 mg/l in the studies of R. A. Omar [65]. *Microbacterium* can provide synchronous removal of heavy metals such as ammonium, nitrites, nitrates, and phosphorus [72], zinc, manganese and copper, manganese, cadmium, and lead [73], which can occur in oil [74].

Thus, our studies have confirmed that *Bacillus cereus* and *Bacillus thuringiensis* strains can remove sulfur from crude oil with a fraction of 0.008 % and 0.364 %, respectively. Further, to enhance this effect, *Bacillus cereus* and *Bacillus thuringiensis* strains have been tested with the use of deep eutectic solvents (DES). To compare the biodesulfurization of crude oil by native strains, strains from the collection of microorganisms *Pseudomonas putida* (B-1827), *Rhodococcus erythropolis* (AC 1039) and *Pseudomonas aeruginosa* (B-5807) were also used.

3.5. Biodesulfurization of high-sulfur oil from the Karazhanbas field in Kazakhstan

To determine the potential for microbial desulfurization of crude oil used mineral-synthetic medium (MSS), as a source of carbon

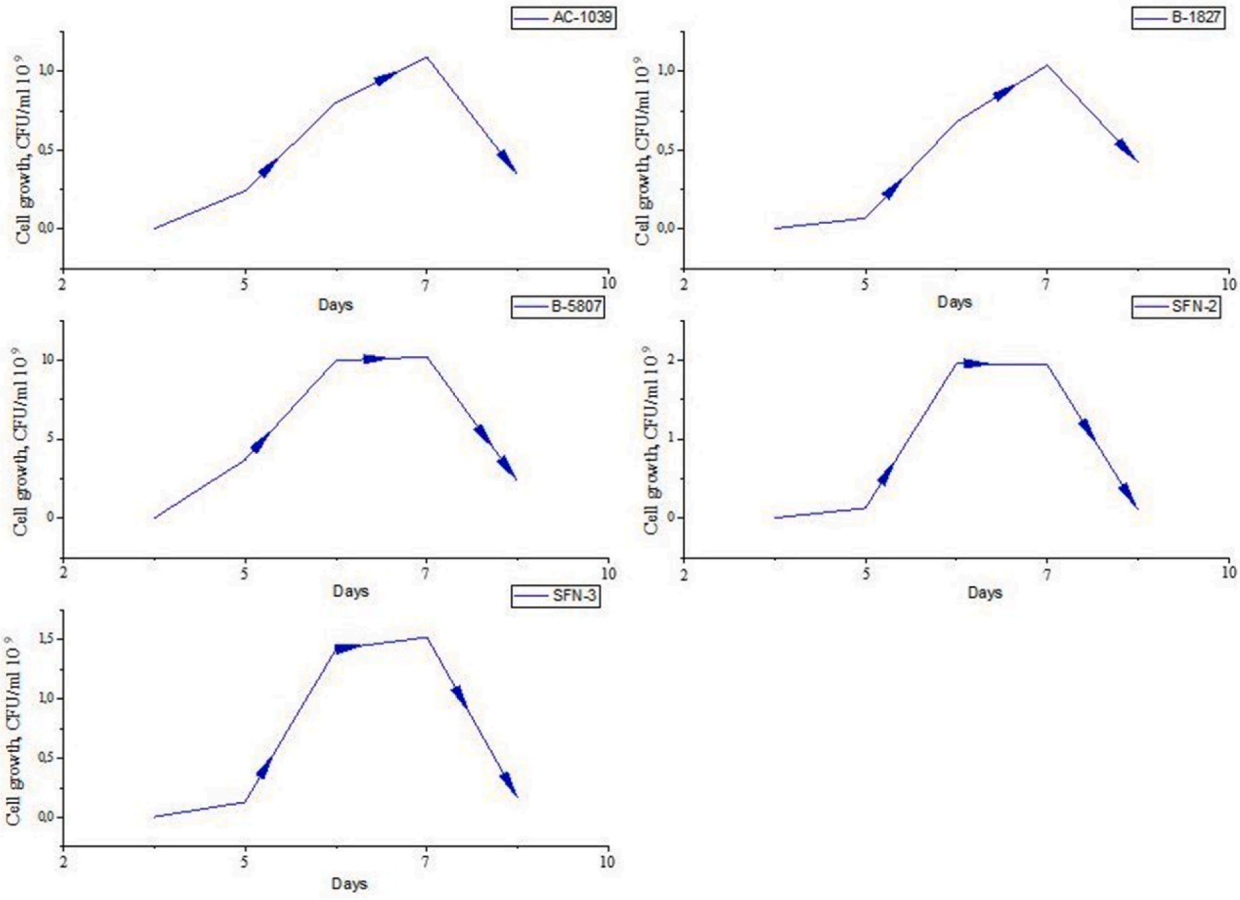


Fig. 6. Growth of microorganisms in a medium with high-sulfur oil, CFU/ml.

and sulfur high-sulfur oil fields "Karazhanbas" as extractants and catalysts of biochemical processes added DES-1 – mixture of betaine ($C_5H_{11}NO_2$) and glycerol ($C_3H_8O_3$) [1:2], DES-2 – a mixture of citric acid ($C_6H_8O_7$) and glycerol ($C_3H_8O_3$) [1:4]. Deep eutectic solvents based on glycerin, betaine, and citric acid protect against osmotic stress, temperature stress, and oxidative stress and play a role in maintaining intracellular pH, provide easy absorption of a nitrogen source [75,76], improve cell growth, promotes salt resistance, stimulating the production of exopolysaccharides [77,78].

Fig. 6 shows the results of the growth of microorganisms in a medium with high-sulfur oil. Figs. 7 and 8 show graphs of the growth of microorganisms in a medium with high-sulfur oil with the addition of DES-1 and DES-2 as extractants and catalysts for biochemical processes. The control options were experiments without microorganisms.

During observations of the dynamics of microbial growth on high–sulfur oil, it was revealed that all crops actively multiply, which means active destruction of the only carbon source - high-sulfur oil. Thus, the maximum increase in the number of cells is observed for the culture of *Pseudomonas aeruginosa B*-5807 by 4 orders of magnitude – from 10^6 to 10^{10} CFU/ml, then for the remaining 4 cultures (*Rhodococcus erythropolis AC 1039, Pseudomonas putida B-1827, Bacillus cereus SFN-2, Bacillus thuringiensis SFN-3*) there is an increase in the content cells by 3 orders of magnitude.

The graphs of Figs. 7 and 8 showed that all the studied bacterial cultures multiply intensively on the medium with DESs from the first day, while there is a slight stimulation of cell growth in comparison with growth without DESs. The maximum increase in the number of cells was also revealed for the culture of *Pseudomonas aeruginosa B*-5807 by 4 orders of magnitude – from 10^6 to 10^{10} CFU/ml. For the remaining 4 cultures of microorganisms (*Rhodococcus erythropolis AC 1039, Pseudomonas putida B-1827, Bacillus cereus SFN-2, Bacillus thuringiensis SFN-3*), an increase in cell content by 3 orders of magnitude is also observed.

It should be noted that for the bacteria *Rhodococcus erythropolis AC 1039* and *Pseudomonas putida B-1827*, an increase in cell growth rate was shown with the addition of both DES-1 and DES-2: there was an increase in the content of cells in the medium by 3 orders of magnitude on day 5, whereas without DES such an indicator was observed only on day 7.

Thus, the addition of DES-1 and DES-2 to a microbe-fermented medium with high-sulfur oil gives a slight stimulation of the cells of all five studied microbial cultures, however, for the bacteria *Rhodococcus erythropolis AC 1039* and *Pseudomonas putida B-1827*, an increase in cell growth rate was shown with the addition of both extractants: an increase in the content of cells in the medium by 3 orders of magnitude is observed on the 5th day, whereas without DES, such an indicator is observed only on the 7th day.

After microbial desulfurization, work was carried out to chemically determine the mass fraction of sulfur (%) in the composition of oil. In the control variants, the sulfur content in the initial nutrient medium with oil was 2.129 %.

The content of the mass fraction of sulfur (%) in the samples without the addition of deep eutectic solvents (DES) by the end of the experiment, on the 10th day, decreased by.

- 32,8 % with *Pseudomonas aeruginosa B5807* (from 2129 % to 1383 %);
- 95,2 % with *Rhodococcus erythropolis AC1039* – (from 2129 % to 0,102 %);
- 27,0 % with *Pseudomonas putida B1827* – (from 2129 % to 1554 %);
- 75,6 % with *Bacillus cereus SFN2* – (from 2129 % to 0,518 %);
- 96,1 % with *Bacillus thuringiensis SFN3* – (from 2129 % to 0,081 %).

The content of the mass fraction of sulfur (%) in the samples with the addition of DES-1 by the end of the experiment, on the 10th day, decreased by.

- 90,0 % with *Pseudomonas aeruginosa B5807*– (from 2129 % to 0,211 %);
- 83,0 % with *Rhodococcus erythropolis AC1039* – (from 2129 % to 0,361 %);
- 17,4 % with *Pseudomonas putida B1827* – (from 2129 % to 1758 %);
- 81,3 % with *Bacillus cereus SFN2* – (from 2129 % to 0,396 %);
- 49,1 % with *Bacillus thuringiensis SFN3*- (from 2129 % to 1082 %).

The content of the mass fraction of sulfur (%) in the samples with the addition of DES-2 by the end of the experiment, on the 10th day, decreased by.

- 96,3 % with *Pseudomonas aeruginosa B5807* – (from 2129 % decreased by 0,0782 %);
- 96,0 % with *Rhodococcus erythropolis AC1039* – (from 2129 % decreased by 0,084 %);
- 28,6 % with *Pseudomonas putida B1827* – (from 2129 % to 1520 %);
- 29 % with *Bacillus cereus SFN2* – (from 2129 % to 0,290 %);
- 57,3 % with *Bacillus thuringiensis SFN3* – (from 2129 % to 0,907 %).

Thus, in the conducted studies, the highest percentage of desulfurization (90 % or more) was noted for strains *Bacillus thuringiensis SFN3* (96.1 %) and *Rhodococcus erythropolis AC1039* (95.2 %) from the control group of experiments. Strains of *Pseudomonas aeruginosa B5807* (90 %) from the experiment with the addition of DES-1, *Pseudomonas aeruginosa B5807* (96.3 %) demonstrate ideal performance as in the studies of Olawumi O. Sadare and Michael O. Daramola [79]. *Rhodococcus erythropolis AC1039* (96 %) from experience with the addition of DES-2. A comparable decrease in sulfur content was previously described for desulfurization of petroleum products using R. erythropolis in studies by Bo Yu and co-authors, which achieved sulfur removal of up to 94.5 % [80].

The results on the chemical content of the mass fraction of sulfur correlate with the results of determining the growth of

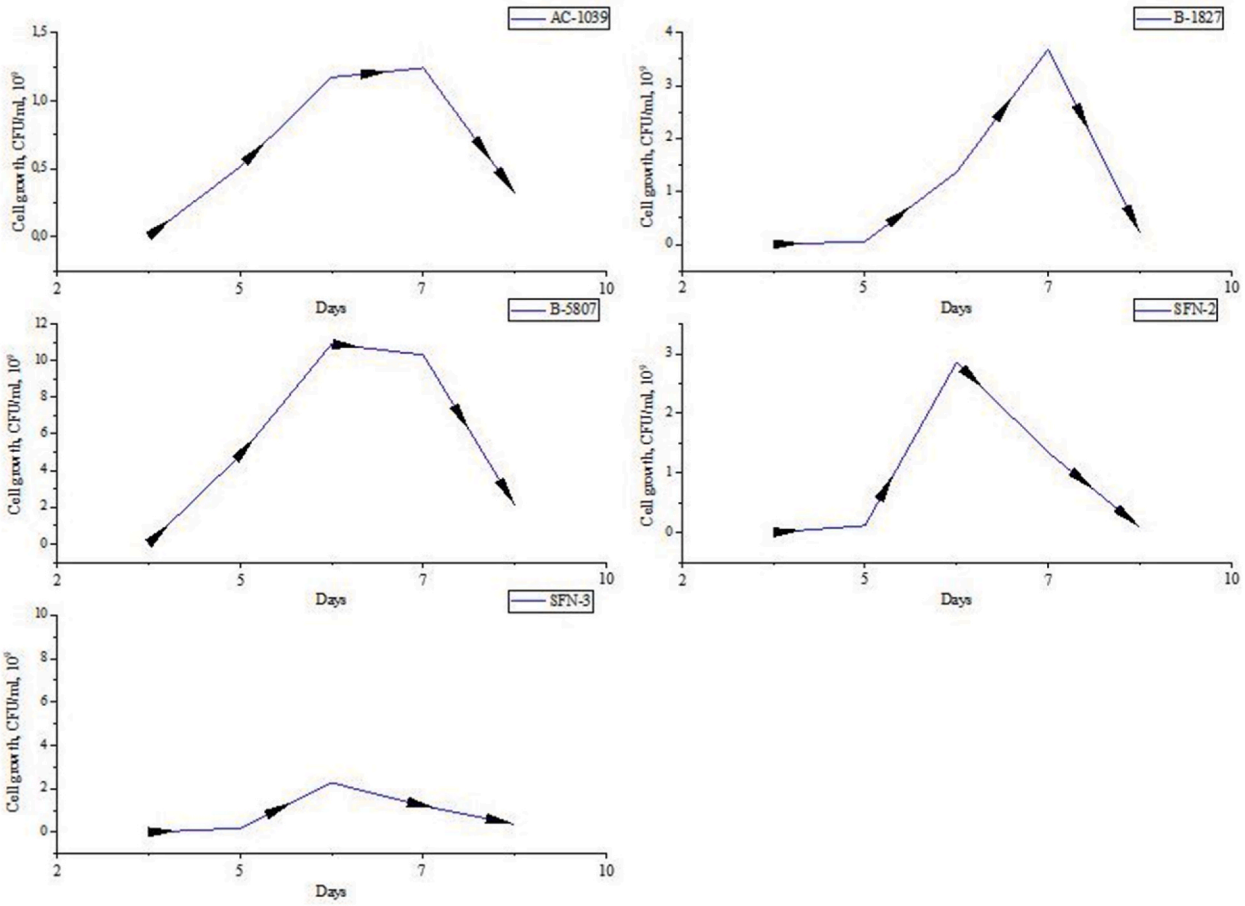


Fig. 7. Growth of microorganisms in a medium with high-sulfur oil with the addition of DES-1 (2 %), CFU/ml.

microorganisms in high-sulfur oil (Figs. 5–7). The study of the graphs showed that, in general, active microbial desulfurization of high-sulfur oil occurs. Thus, the oil content in the initial nutrient medium is 2129 %, and the addition of extractants DES-1 and DES-2 intensifies the active process of microbial desulfurization. Based on the presented data, it can be concluded that a low percentage of sulfur content at the end of the experiment was observed for cultures of *Pseudomonas aeruginosa B5807* with the addition of DES-2 - 0.0782 %, *Bacillus thuringiensis SFN-3* - 0.081 %, *Rhodococcus erythropolis AC1039* with the addition of DES-2 - 0.084 %. The maximum indicators of microbial desulfurization of oil were achieved: with the addition of DES-2 - 0,0782-1520 %; when adding a DES-1 - 0,211–1758 %; and without the addition of DES, the microbial desulfurization index was 0.081–1.554 %.

4. Conclusion

Studies on biodesulfurization of oil fields in Kazakhstan were based on work with samples of high–sulfur oils taken from the Karazhanbas (1.6–2.2 %) and Buzachi (1.78 %) fields of the Mangystau region and low-sulfur oil (0.10–0.14 % by weight) from the Kumkol field of the Ulytau region.

The isolation of sulfate-oxidizing and sulfate-reducing bacteria from crude oil showed that microbiological activity in crude oil was recorded in the Kumkol and Karazhanbas fields, and an order of magnitude lower in the Buzachi oil field, while all isolated microorganisms showed colony growth only on the 3rd day of cultivation.

12 aerobic bacterial cultures were isolated from oil samples on a universal MPA medium, of which in the testing process:

- on a medium containing high-sulfur oil as the only carbon source, 9 strains with active and moderate growth on a medium with high-sulfur oil were selected: *Bacillus paramycoides (SFN-1), Bacillus cereus (SFN2), Bacillus thuringiensis (SFN3), Bacillus haynesii (F1), Bacillus zhangzhouensis (F2), Peribacillus simplex (E-1), Bacillus subtilis (H1), Microbacterium* sp. *(H2), Bacillus siamensis (H4)*;
- two strains were selected on the medium with Cr(VI), which were the most resistant, since relatively high cell counts were recorded in the medium during 24, 48 and 72 h of cultivation, in particular, *Bacillus paramycoides SFN-1* (0.284; 0.264 and 0.213 units of cells in the medium with Cr (VI)) and *Bacillus cereus SFN-2* (0.266; 0.191 and 0.161 units of cells in a medium with Cr (VI));

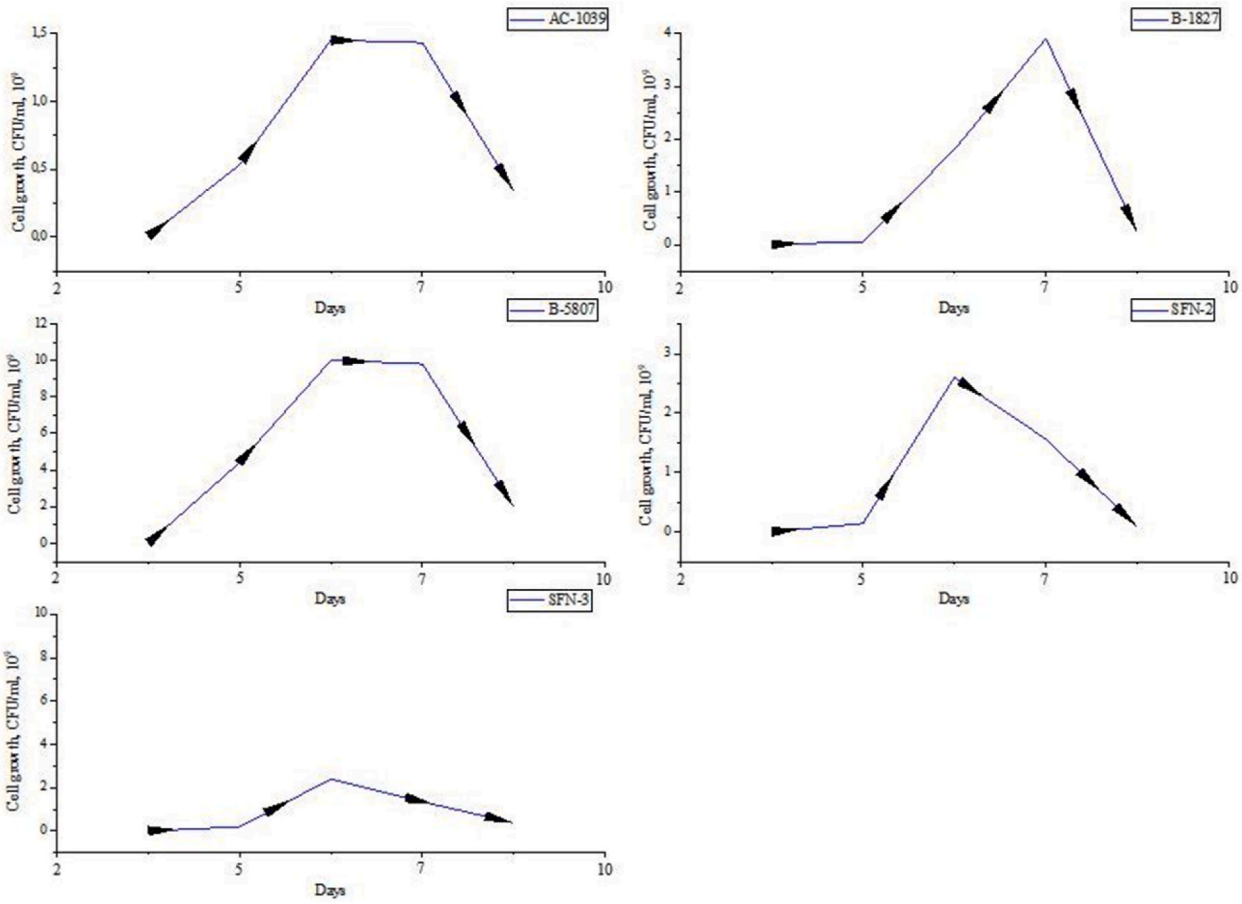


Fig. 8. Growth of microorganisms in a medium with high-sulfur oil with the addition of DES- 2 (2 %), CFU/ml.

– at the end of the experiment, on the 5th day, *Bacillus cereus (SFN2)* and *Bacillus thuringiensis (SFN3)* strains were selected on the medium concerning sulfur in the composition of crude high-sulfur oil, which showed sulfur-oxidizing abilities: the content of the mass fraction of sulfur decreased by 0.008 % and 0.364 % compared with those obtained in sterile the control group of oil, respectively.

During biodesulfurization of high-sulfur oil from the Karazhanbas field in Kazakhstan, deep eutectic solvents were used for comparison with native strains (*Bacillus cereus SFN2, Bacillus thuringiensis SFN3*), strains from the collection of microorganisms *Pseudomonas putida (B-1827), Rhodococcus erythropolis (AC 1039)* and *Pseudomonas aeruginosa (B -5807)*, while the content of the mass the proportion of sulfur (%) by the end of the experiment, on the 10th day, decreased as much as possible.

– in samples without the addition of deep eutectic solvents (DES), 95.2 % with *Rhodococcus erythropolis AC1039* – (from 2.129 % to 0.102 %) and 96.1 % with *Bacillus thuringiensis SFN3* – (from 2.129 % to 0.081 %);
– in samples with the addition of DES-1, 90.0 % with *Pseudomonas aeruginosa B5807* – (from 2.129 % to 0.211 %), 81.3 % with *Bacillus cereus SFN2* – (from 2.129 % to 0.396 %) and 49.1 % with *Bacillus thuringiensis SFN3* – (from 2.129 % to 1.082 %);
– in samples with the addition of DES-2 by 96.3 % with *Pseudomonas aeruginosa B5807* – (from 2.129 % decreased by 0.0782 %), by 96.0 % with *Rhodococcus erythropolis AC1039* – (from 2.129 % decreased by 0.084 %), by 29 % with *Bacillus cereus SFN2* – (from 2.129 % to 0.290 %) and by 57.3 % with *Bacillus thuringiensis SFN3* – (from 2.129 % to 0.907 %).

Thus, it can be concluded that the native bacterial strains selected during the study have high desulfurization activity without the addition of deep eutectic solvents (*Bacillus thuringiensis SFN3*), with the addition of DES-1 (*Bacillus cereus SFN2*) and with the addition of DES-2 (*Bacillus thuringiensis SFN3*).

CRediT authorship contribution statement

A.O. Akimbek: Methodology, Formal analysis. **G.A. Jamalova:** Supervision, Data curation, Conceptualization. **A.K.**

Yernazarova: Project administration, Methodology, Investigation, Conceptualization. **G.K. Kaiyrmanova:** Conceptualization. **B.K. Yelikbayev:** Visualization, Validation, Resources. **M.C. Pagano:** Supervision, Formal analysis, Data curation. **A.G. Zazybin:** Resources, Conceptualization. **Kh S. Rafikova:** Writing – review & editing, Writing – original draft, Conceptualization.

Data availability statement

Data will be made available on request. For requesting data, please write to the corresponding author.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Khadichakhan Rafikova reports financial support was provided by K. I. Satbayev Kazakh National Research Technical University. Khadichakhan Rafikova reports a relationship with Satbayev University that includes: employment. Khadichakhan Rafikova has patent - pending to -. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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